

Bone Sialoprotein, Matrix Metalloproteinase 2, and $\alpha_v\beta_3$ Integrin in Osteotropic Cancer Cell Invasion

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Background: Bone sialoprotein (BSP) interacts separately with both matrix metalloproteinase 2 (MMP-2) and integrin $\alpha_v\beta_3$ and is overexpressed in many metastatic tumors. Its role in tumor biology, however, remains unclear. We investigated whether BSP enhances cancer cell invasiveness by forming a trimolecular complex with MMP-2 and cell-surface integrin $\alpha_v\beta_3$. **Methods:** Invasiveness of breast, prostate, lung, and thyroid tumor cell lines was measured with a modified Boyden chamber assay. Binding and co-localization of BSP, MMP-2, and integrin $\alpha_v\beta_3$ were investigated with immunoprecipitation and *in situ* hybridization. All statistical tests were two-sided. **Results:** Treatment with BSP increased invasiveness of many breast, prostate, lung, and thyroid cancer cells through Matrigel in a dose-dependent manner. BSP at 50 nM increased the invasiveness of SW-579 thyroid cancer cells (95.2 units, 95% confidence interval [CI] = 90.4 to 100 units) by approximately 10-fold compared with that of untreated control SW-579 cells (9.1 units, 95% CI = 5.7 to 12.5 units) ($P < .001$). Addition of an inactive mutated BSP, in which BSP's integrin-binding RGD tripeptide was altered, or addition of integrin $\alpha_v\beta_3$ -blocking antibodies resulted in invasiveness equivalent to that of untreated cells. Inhibiting cellular MMP-2 activity with chemical inhibitors or a specific antibody also blocked BSP-enhanced invasiveness. Osteopontin and dentin matrix protein 1, proteins related to BSP that also bind integrin $\alpha_v\beta_3$ and form complexes with other MMPs (but not MMP-2), did not enhance invasiveness. Immunoprecipitation showed that a complex containing BSP, integrin $\alpha_v\beta_3$, and MMP-2 formed *in vitro*. Addition of BSP increased the amount of MMP-2 bound by cells in an integrin-dependent fashion. Co-expression of BSP, integrin $\alpha_v\beta_3$, and MMP-2 in papillary thyroid carcinoma cells was shown by *in situ* hybridization. **Conclusion:** Cancer cells appear to become more invasive when BSP forms a cell-

surface trimolecular complex by linking MMP-2 to integrin $\alpha_v\beta_3$. [J Natl Cancer Inst 2004;96:956–65]

The members of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family of secreted proteins contain an integrin-binding tripeptide (arginine-glycine-aspartate, or RGD) and several conserved serine/threonine (Ser/Thr) phosphorylation and N-glycosylation sites. SIBLINGs include bone sialoprotein (BSP), osteopontin, dentin matrix protein 1 (DMP1), dentin sialophosphoprotein, and matrix extracellular phosphoglycoprotein (1). Genes for all of these SIBLINGs are clustered within a 375 000 base-pair (bp) region of human chromosome 4 (chromosome 5 in the mouse) (2). SIBLING expression is normally restricted to skeletal tissues in adults but also includes trophoblasts during embryonic development (3–5). Osteopontin is an exception, being expressed in normal kidney (6), lactating breast (7), and immune cells (8). BSP normally interacts only with cell-surface integrins, such as integrin $\alpha_v\beta_3$ (also known as the vitronectin receptor), whereas osteopontin and DMP1 bind to both integrins and CD44 (9–13).

BSP is overexpressed by many malignant tissues, including breast (14), prostate (15), lung (16), and thyroid (17) cancers and

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melanoma (18). BSP expression has been associated with clinical severity and poor survival among patients with breast cancer (19) or with prostate cancer (15). Recently developed serum immunoassays for BSP and osteopontin show that serum from patients with breast, lung, colon, or prostate cancer had statistically significantly elevated levels of BSP and/or osteopontin (20). However, the role of BSP in these cancers is unclear.

Matrix metalloproteinases (MMPs), a class of zinc-dependent endopeptidases, are collectively capable of digesting all extracellular matrix components. In addition to their role in normal tissue development and remodeling, MMPs appear to play major roles in tumor cell invasion and metastasis (21). Although the mechanism by which tumors invade surrounding tissues is not completely understood, MMPs may play an important role by removing physical barriers to invasion. In particular, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) degrade extracellular matrix macromolecules in basement membranes and other interstitial connective tissues (22). Active MMP-2 can localize to the cell surface by binding directly to integrin $\alpha_v\beta_3$ (23), and proteolytically active MMP-9 can associate with CD44 (24), thereby focusing proteolytic activity on the cell membrane at the leading edge of the invasive cell.

The integrins are a family of transmembrane receptor proteins composed of heterodimeric complexes of α and β chains (25). There are 18 α and eight β chains, and these chains can dimerize to form at least 25 different complexes, each binding to a specific set of ligands. For example, integrin $\alpha_v\beta_3$ binds to BSP, osteopontin, and DMP1. In addition to regulating cell adhesion to the extracellular matrix, integrins modulate many cellular processes including proliferation, apoptosis, migration, and invasiveness by activating various signaling pathways (26). Some integrins are overexpressed in malignant tumors. For example, integrin $\alpha_v\beta_3$ is expressed at the invasive front of malignant melanoma cells and on angiogenic blood vessels (27). The level of integrin $\alpha_v\beta_3$ expression in breast cancers is associated with the aggressiveness of the disease (28).

It is generally accepted that latent pro-MMPs are enzymatically activated by removal of their inhibitory propeptide. BSP, osteopontin, and DMP1 bind with nanomolar affinity to the latent and active forms of MMP-2, MMP-3, and MMP-9, respectively. When purified SIBLINGs are incubated with their pro-MMP partners, increased proteolytic activity is detected (29). Therefore, we hypothesize that one or more SIBLINGs increase the invasiveness of cancer cells by interacting with their specific MMP and integrin partners. To test this hypothesis, we used a modified Boyden chamber cell invasion assay, as described previously (30), to measure the invasiveness of various cancer cell lines through a layer of Matrigel (a mixture of basement membrane components), and we determined whether BSP increases the invasiveness of cancer cells by forming a trimolecular complex in which BSP acts as a bridge to link MMP-2 to integrin $\alpha_v\beta_3$.

MATERIALS AND METHODS

Materials

Human breast cancer cell lines MDA-MB-231 (HTB-26), MDA-MB-435S (HTB-29), BT-474 (HTB-20), and MCF-7 (HTB-22); human prostate cancer cell lines PC-3 (CRL-1435), LNCaP (CRL-1740), and DU-145 (HTB-81); human thyroid

cancer cell line SW-579 (HTB-107); human lung cancer cell line NCI-H520 (HTB-182); and human osteosarcoma cell lines SK-ES-1 (HTB-86), SaOS-2 (HTB-85), and MG-63 (CRL-1427) were obtained from the American Type Culture Collection (Manassas, VA). The mouse fibroblastic cell line NIH 3T3 was provided by Dr. Hynda Kleinman (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). Fetal bovine serum was purchased from Equitech-Bio (Kerrville, TX). RPMI-1640 medium, L-glutamine, 2-mercaptoethanol, sodium pyruvate, modified Eagle medium (MEM) nonessential amino acids, Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), Versene (0.53 mM EDTA in PBS), and 10% zymogram gelatin gels were from Invitrogen (Carlsbad, CA). Matrigel was from Collaborative Biotech (Bedford, MA; provided by Dr. Hynda Kleinman). Transwell inserts and companion plates were purchased from BD Biosciences Discovery Labware (Bedford, MA). Calcein acetoxymethyl ester dye and the Alexa Fluor 488 protein labeling kit were purchased from Molecular Probes (Eugene, OR). Mouse anti-human vitronectin receptor monoclonal antibody immobilized on immunoaffinity gel matrix (GEM1976), vitronectin receptor complex in Triton X-100 (CC1018), and mouse anti-MMP-2 monoclonal antibody (MAB 13435) were obtained from CHEMICON International (Temecula, CA). Pro-MMP-2 and active MMP-2 were from Oncogene Research Products (Boston, MA). pBluescript II KS vector was purchased from Stratagene (La Jolla, CA). Digoxigenin labeling mixture was obtained from Roche Biochemicals (Indianapolis, IN). The *in situ* hybridization kit, which included 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; product SH-3018-01), was from InnoGenex (San Ramon, CA). 1,10-Phenanthroline was from Sigma Chemical Co. (St. Louis, MO). MMP-2 inhibitor I, *cis*-9-octadecenoyl-N-hydroxylamideleoyl-N-hydroxylamide, and anti-integrin $\alpha_v\beta_3$ monoclonal antibody (LM609, MAB 1976Z) were obtained from Calbiochem (San Diego, CA). Rhodamine Red-conjugated AffiniPure goat anti-rabbit immunoglobulin G (IgG; heavy- and light-chain) and Cy2-conjugated AffiniPure goat anti-mouse IgG (heavy- and light-chain) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Vectashield mounting medium for fluorescence microscopy with 4',6-diamidino-2-phenylindole (DAPI; product H-1200) was obtained from Vector Laboratories (Burlingame, CA). The ProbeQuant G-50 microcolumn was from Amersham Pharmacia Biotech (Piscataway, NJ). The Microcon YM-30 centrifugal filter device was from Millipore (Bedford, MA). *In situ*-ready human thyroid papillary adenocarcinoma serial paraffin sections (product 70452-3) were purchased from Novagen, (Madison, WI).

Cell Culture

The human cancer cell lines used, as described above, were first grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 mM 2-mercaptoethanol, 2 mM sodium pyruvate, and 0.1 mM MEM nonessential amino acids in a humidified atmosphere of 5% CO₂/95% air at 37 °C. When the cells were approximately 80% confluent, they were used in the experiments described below or subcultured for up to 20 passages at a split ratio of 1:10.

SIBLING Production and Purification

Recombinant BSP, BSP-KAE (BSP in which the RGD sequence was replaced with the sequence KAE), osteopontin, and DMP1 with posttranslational modifications, including glycosylation, sulfation, and possibly phosphorylation, were made as described previously (9,11). Briefly, adenoviral constructs containing full-length human BSP (31), BSP-KAE (9), osteopontin (32), or bovine DMP1 (33) were subcloned into high-expression, replication-deficient adenovirus type 5 under the control of the elongation factor 1 (EF-1) promoter for BSP or the cytomegalovirus (CMV) promoter for BSP-KAE, osteopontin, and DMP1. The BSP-KAE constructs were made by *in situ* mutagenesis in pBluescript; the entire insert was checked for fidelity and then shuttled to the adenovirus plasmid (34). The adenoviruses were selected, purified, and expressed as previously described (9). Recombinant SIBLINGS were generated by infecting mid-passage subconfluent normal human bone marrow stromal fibroblasts (gift from Dr. P. Gehron Robey, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). Harvested serum-free medium was subjected to anion-exchange chromatography, as described (9,11), to isolate SIBLINGS. The purity of each SIBLING was greater than 95%, as measured after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Modified Boyden Chamber Cell Invasion Assay

Invasiveness of each cancer cell line was measured by using a UV-opaque transwell polycarbonate membrane insert with a diameter of 6.4 mm and pore size of 8 μm in a modified Boyden chamber cell invasion assay. Transwell inserts were placed in a 24-well plate, precoated with Matrigel (5–10 μg in 50 μL per well), and dried overnight in a laminar airflow hood. Preconfluent cells were removed from culture dishes with 0.53 mM EDTA in PBS, washed twice in HBSS, and resuspended in serum-free RPMI-1640 culture medium at a final density of 4×10^5 cells per milliliter. Quadruplicate cultures of cells were briefly pretreated in a final volume of 250 μL of serum-free medium (containing 0.1% bovine serum albumin) with either buffer or SIBLINGS in 1.5-mL Eppendorf microcentrifuge tubes for 10 minutes and then placed in the upper compartment of a Boyden chamber. In some cases, cells were first treated for 20 minutes with inhibitors, blocking antibodies, or isotype control IgGs in the tube and then placed in the upper chamber. In the latter cases, buffer or recombinant SIBLING was then added directly to the chamber. To induce migration through the Matrigel layer, the lower chambers were filled with 750 μL of serum-free medium conditioned by mouse NIH 3T3 fibroblastic cells and containing 0.1% bovine serum albumin. Cells were then incubated in a humidified incubator at 37 °C for 6–24 hours, depending on the cell line. Cells that had not migrated through the barrier were removed from the top compartment, and inserts were moved to another 24-well plate in which each well contained 0.5 mL of the fluorescent dye calcein acetoxymethyl ester at 4 $\mu\text{g}/\text{mL}$. The plate was incubated at 37 °C for 45 minutes to allow the living cells to take up and activate the dye, and then the fluorescent intensity was read from the bottom of the insert with a fluorescence plate reader (Wallac 1420 VICTOR² Multilabel Reader; PerkinElmer Life Sciences, Boston, MA). Fluorescence intensity was proportional to the number of cells migrating to the bottom of the UV-opaque membrane.

Immunoprecipitation Experiments

Commercial mouse anti-human vitronectin receptor (integrin $\alpha_v\beta_3$) monoclonal antibody immobilized on immunoaffinity gel matrix (i.e., beads) was washed three times in ice-cold Triton buffer (TB; 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.2% Triton X-100, 2 mM MgCl_2 , and 0.1 mM CaCl_2) and incubated in 1 mL of TB containing 1% bovine serum albumin at 4 °C for 30 minutes with gentle shaking. After washing three times with 1 mL of ice-cold TB, the beads were gently shaken with or without 10 μg of integrin $\alpha_v\beta_3$ in 50 μL of TB at 4 °C for 10 minutes. The beads were then pelleted, the liquid was carefully removed, and the beads were washed in 1 mL of TB. The beads were then resuspended in 1 mL of buffer and separated into aliquots. An aliquot was gently shaken with buffer alone or buffer containing 500 nM BSP or 500 nM BSP-KAE (in a final volume of 50 μL) at 4 °C for 10 minutes. The beads were then pelleted, washed in 1 mL of TB, and incubated in 50 μL of TB containing 1 μg of pro-MMP-2 or 1 μg of active MMP-2 at 4 °C for 10 minutes. The beads were pelleted and washed with 1 mL of TB. The MMPs were eluted from the beads with 80 μL of 1 \times SDS sample buffer (2.5 mL of 0.5 M Tris-HCl [pH 6.8], 2 mL of glycerol, 4 mL of 10% (wt/vol) SDS, and 0.5 mL of 0.1% bromophenol blue, adjusted to 20 mL with distilled water) and resolved by electrophoresis on a 10% gelatin zymogram gel.

SDS-Polyacrylamide Gel Electrophoresis and Zymography

Samples in zymogram gel sample buffer were loaded on a 10% gelatin zymogram gel, subjected to electrophoresis, and processed as recommended by the manufacturer. Resulting Coomassie-stained gels were visualized with an EagleEye II imaging system (Stratagene, La Jolla, CA) by dynamic integrated exposure with an initial integration time of 3 seconds and an increment of 3 seconds (the camera sums frames of 1/30 second for a 3-second period, sends the image to the computer, collects light for 6 seconds, sends the image to the computer, and continues in this progression until integration is stopped).

Labeling of Purified Human Active MMP-2 and Pro-MMP-2 With Alexa 488 Dye

Latent (pro-MMP-2) or active MMP-2 was fluorescently labeled with the Alexa Fluor 488 protein labeling kit according to the manufacturer's protocol but was adjusted to the smaller amounts of protein being labeled. Briefly, shipping buffer from 50 μg of pro-MMP-2 or 50 μg of active MMP-2 was exchanged for the reaction buffer (PBS) on ProbeQuant G-50 microcolumns, and the resulting mixture was concentrated to approximately 50 μL with a prewashed Microcon YM-30 centrifugal filter device. Sodium bicarbonate (0.1 M, 5 μL) was added to raise the pH to 7.5–8.5 for efficient labeling. All steps were performed at 4 °C. The reactive dye was dissolved in 0.5 mL of PBS containing 0.1 M sodium bicarbonate, 50 μL of Alexa Fluor 488 dye was added to the MMP-2 solution, and the reaction mixture was stirred at 4 °C for 2 hours. The labeled MMP-2 protein was then separated from the unreacted dye on ProbeQuant G-50 microcolumns (in PBS) and stored as aliquots at –80 °C until use.

Flow Cytometry

Cells were detached from culture dishes with PBS containing 0.53 mM EDTA, washed twice in HBSS, and then incubated at

2×10^6 cells per milliliter with buffer alone or buffer containing 500 nM BSP or 500 nM BSP-KAE at room temperature for 10 minutes. For the studies involving the blocking anti-integrin $\alpha_v\beta_3$ antibody, cells were incubated with buffer alone or buffer containing anti-integrin $\alpha_v\beta_3$ antibody (LM609, 4 $\mu\text{g}/\text{mL}$) or isotype control IgG₁ (4 $\mu\text{g}/\text{mL}$) at room temperature for 10 minutes, and then the mixture was incubated with 500 nM BSP for 10 minutes. In the final step for all samples, cells were pelleted at 225g for 10 minutes at room temperature, washed once in HBSS, and then incubated at room temperature with Alexa Fluor 488-labeled purified human pro-MMP-2 at 1 $\mu\text{g}/\text{mL}$ or active MMP-2 at 1 $\mu\text{g}/\text{mL}$ for 10 minutes. The cells were pelleted, washed once, resuspended in HBSS, and analyzed immediately with a FACSCalibur cell sorter equipped with a 488-nm argon laser and Cellquest software (BD Pharmingen, Bedford, MA).

Fluorescent Immunocytochemistry

To localize BSP, MMP-2, and integrin $\alpha_v\beta_3$ on individual cells, 1 mL containing 1×10^3 SW-579 cells was placed in each well of a two-well chamber slide and incubated at 37 °C for 24 hours. The cells were then washed with serum-free RPMI-1640 medium and incubated in this medium at 37 °C with no additions, with 100 nM BSP-KAE, or with 100 nM BSP for 24 hours. The cells were then washed and incubated at 37 °C with recombinant pro-MMP-2 (1 $\mu\text{g}/\text{mL}$ per well) for 20 minutes. After three washes in PBS, the cells were fixed in absolute ethanol at 4 °C for 30 minutes, washed three times in PBS, and incubated in PBS with affinity-purified human anti-BSP polyclonal antibody (LF-84) and mouse anti-MMP-2 monoclonal antibody at the same time at 4 °C for 24 hours. The cells then were washed and incubated with Rhodamine Red-coupled AffiniPure goat anti-rabbit IgG and Cy2-coupled AffiniPure goat anti-mouse IgG secondary antibodies at room temperature for 30 minutes. The slides were detached from the chamber, washed three times with PBS, and immediately mounted in Vectashield mounting medium for fluorescence with DAPI for nuclear staining under a coverslip. The samples were analyzed with a fluorescence microscope that could simultaneously visualize both dye signals.

In Situ Hybridization

To generate strand-specific probes for *in situ* hybridization, a polymerase chain reaction-amplified human MMP-2 cDNA fragment (317 bp) was subcloned into the *Bam*HI site of pBlue-script II KS vector. The oligonucleotides for amplification of the MMP-2-specific probes were 5'-ATTAGGATCCGGTCACAGCTACTTCTTCAAG-3' (forward) and 5'-ATATGGATCCGCCTGGGAGGAGTACAG-3' (reverse). The BSP template was the full-length human BSP cDNA B6-5g (31). The human integrin α_v cDNA (1200 bp) insert originally cloned into the pUC12 vector was released with *Eco*RI and *Hind*III (35), and human integrin β_3 cDNA (2275 bp) originally cloned into the pUC12 vector (36) was released with *Eco*RI. Both cDNA inserts were then subcloned in pBluescript II KS, a vector that contains the T3 and T7 RNA polymerase promoters for RNA probe synthesis. After the plasmids were linearized with the appropriate restriction enzymes, the probes were labeled with a digoxigenin-labeling mixture (1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP, and 0.35 mM DIG-11-UTP [digoxigenin coupled to UTP at position 11], pH 7.5) to produce the specific digoxigenin-

labeled single-stranded antisense and sense RNA fragments. *In situ* hybridization for thyroid papillary carcinoma serial sections was carried out with the InnoGenex *in situ* hybridization BCIP/NBT kit according to the manufacturer's instructions with minor modifications. Slides were deparaffinized in xylene and rehydrated through a graded ethanol series. After a 10-minute incubation in the kit's proteinase K solution, the slides were fixed in 1% formaldehyde for 10 minutes. Approximately 50 μL of hybridization buffer containing pre-titrated digoxigenin-labeled RNA probes (antisense or sense) were applied to each slide. The hybridization reaction included a 3-minute denaturation at 80 °C followed by overnight incubation at 37 °C. After hybridization, washes at room temperature consisted of rinsing with $2 \times$ PBS to remove the coverslip, followed by one 10-minute wash and two 5-minute washes in PBS. The sections were then treated with antibody-blocking solution (InnoGenex, product BS-1310-06) for 5 minutes at room temperature, and the blocking agent was gently removed. Biotinylated mouse anti-digoxigenin monoclonal antibody (InnoGenex, product AS-3000-06) was then applied to the sections for 5 minutes at 37 °C, washed for two 5-minute periods in PBS, and then incubated at 37 °C with alkaline phosphatase streptavidine conjugate (provided by the manufacturer) for 5 minutes. After washing twice with PBS, activation buffer was then applied to each section for 1 minute before incubating in BCIP/NBT substrate chromogen solution until satisfactory color reaction was observed (approximately 15 minutes). Sections were then counterstained with nuclear fast red, dehydrated through a graded series of alcohol and xylene, and mounted under a coverslip. Sections were photographed with an AxioCam MR-MRGrab camera imaging system (Carl Zeiss Vision, Munchen, Germany), which included an Axio-plan2 microscope, an AxioCam MRm camera, and AxioVision 3.1 software.

Statistical Analysis

Data are the mean of quadruplicate determinations and its 95% confidence interval (CI). Each experiment was repeated at least two times. In each case, data from a single representative experiment are shown. Multiple comparisons were performed with a one-way analysis of variance followed by Dunnett's test for treatment versus control comparisons. Pairwise comparisons were carried out by performing a nonparametric Mann-Whitney *U* test. In each analysis, differences were considered statistically significant for *P* values less than .05. All statistical tests were two-sided.

RESULTS

BSP and Invasiveness of Cancer Cells *In Vitro*

Recent reports (14–20) that BSP is elevated in tumors and serum from patients with breast, prostate, lung, or thyroid cancers prompted us to investigate whether BSP has a role in the invasion of cancer cells. Invasiveness was measured with a modified Boyden chamber cell invasion assay. Treatment with BSP caused dose-dependent increases in the invasiveness of the breast cancer cell lines MDA-MB-231, MDA-MB-435S, and MCF-7; prostate cancer cell lines PC-3 and DU-145; lung cancer cell line NCI-H520; and thyroid cancer cell line SW-579. Results from a representative cell line for each cancer type are shown in Fig. 1. MDA-MB-231 cells showed a clear dose-

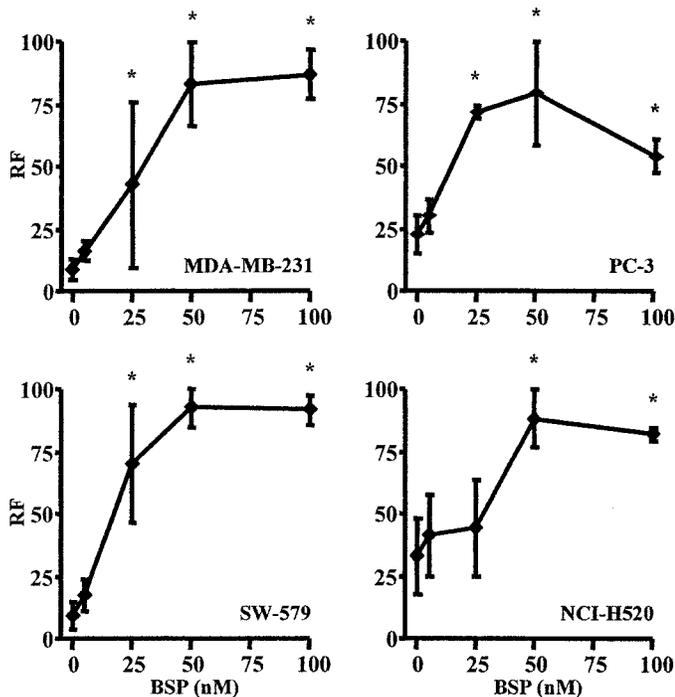


Fig. 1. Bone sialoprotein (BSP) and the invasion of selected cancer cell lines *in vitro*. BSP-treated breast (MDA-MB-231), prostate (PC-3), thyroid (SW-579), and lung (NCI-H520) cancer cells were placed in the Matrigel-coated upper chamber of a Boyden chamber with a UV-opaque transwell insert. The lower chambers contained serum-free conditioned medium as a chemoattractant. The cells were incubated at 37 °C for 6–24 hours. Invasive cells that penetrated the Matrigel artificial basement membrane into the lower chamber were detected by calcein acetoxymethyl ester (AM) fluorescent dye, which is activated by living cells. The relative fluorescence (RF) in the lower chamber corresponds directly to the number of cells that have migrated through the Matrigel. Data are the means of quadruplicate samples, and **error bars** are the 95% confidence intervals. A *P* value of <.001 was obtained for multiple comparisons within each panel, by use of one-way analysis of variance. Each treatment group was also individually compared with the control untreated group by use of Dunnett's test. **P*<.001 compared with untreated cells by Dunnett's test. All statistical tests were two-sided.

response increase in their invasiveness through a Matrigel barrier, with a maximum increase of approximately 10-fold at 100 nM BSP (93.1 units [U; 1 U represents 1% of the maximum number of cells invaded], 95% CI = 86.6 to 99.6 U) compared with that of untreated cells (9.5 U, 95% CI = 6.8 to 12.2 U) (*P*<.001) (Fig. 1). MDA-MB-435S cells showed an approximately twofold increase at 100 nM BSP (84.7 U, 95% CI = 69.4 to 100.0 U) compared with that of untreated cells (43.7 U, 95% CI = 36.8 to 50.6 U) (*P*<.001). In addition, MCF-7 cells, which are usually not aggressive in Boyden chamber cell invasion assays, showed a statistically significant approximately ninefold increased invasiveness after treatment with 100 nM BSP (79.5 U, 95% CI = 59.0 to 100.0 U) compared with untreated cells (8.5 U, 95% CI = 3.0 to 14.0 U) (*P*<.001).

Addition of recombinant human BSP to cultured prostate cancer cell lines PC-3 and DU-145 increased their invasiveness. The invasiveness of PC-3 cells increased more than threefold after the addition of 50 nM BSP (86.2 U, 95% CI = 72.4 to 100.0 U) compared with untreated cells (25.0 U, 95% CI = 19.7 to 30.3 U) (*P*<.001) (Fig. 1). Treatment with 100 nM BSP caused an approximately 17-fold increase in the invasiveness of DU-145 cells (84.6 U, 95% CI = 69.3 to 99.9 U) compared with

untreated cells (5.0 U, 95% CI = 4.7 to 5.3 U) (*P*<.001) but caused no change in the invasiveness of another prostate cancer cell line, LNCaP (data not shown). BSP at 50 nM enhanced the invasiveness of the less aggressive lung squamous cell carcinoma NCI-H520 cells by approximately 2.7-fold (92.5 U, 95% CI = 85.0 to 100.0 U) compared with untreated control NCI-H520 cells (34.2 U, 95% CI = 24.4 to 44.0 U) (*P*<.001) (Fig. 1). The thyroid squamous cell carcinoma SW-579 cells responded maximally to the addition of 50 nM BSP with an approximately 10-fold increase in invasiveness (95.2 U, 95% CI = 90.4 to 100.0 U) compared with untreated cells (9.1 U, 95% CI = 5.7 to 12.5 U) (*P*<.001) (Fig. 1). BSP did not increase the invasiveness of the osteosarcoma cell lines SK-ES-1, SaOS-2, and MG-63 (data not shown). None of the cell lines used in these experiments expressed an appreciable level of BSP, as measured by northern blot hybridization (data not shown).

The increase in the invasiveness of these cancer cell lines was specific to BSP, because the same dose range of osteopontin and DMP1, the two other members of the SIBLING family that can support cell attachment but cannot bind to MMP-2, did not increase the invasiveness of any of the cell lines tested (data not shown).

BSP-Enhanced Invasion, Integrin $\alpha_v\beta_3$, and MMP-2

The same invasiveness studies were performed with BSP-KAE, a recombinant BSP protein whose integrin-binding RGD sequence was replaced with the chemically similar but inactive tripeptide KAE. Treatment with BSP-KAE did not increase the invasiveness of any cell line that had previously responded to BSP compared with the invasiveness of untreated cells. Results from representative breast, prostate, thyroid, and lung cancer cell lines are shown in Fig. 2.

BSP binds to the vitronectin receptor, which is also known as $\alpha_v\beta_3$ integrin (37). As shown in Fig. 3, when SW-579 thyroid cancer cells were pretreated with isotype control IgG₁ and then treated with BSP, their invasiveness increased about fivefold (90.3 U, 95% CI = 80.6 to 100.0 U) compared with untreated cells (19.4 U, 95% CI = 16.0 to 22.8 U). However, when cells were first incubated with the same amount of the integrin $\alpha_v\beta_3$ monoclonal antibody LM609, which blocks RGD binding, and then treated with BSP, invasiveness (21.7 U, 95% CI = 10.9 to 32.5 U) was similar to that of untreated cells (19.4 U, 95% CI = 16.0 to 22.8 U). Thus, the BSP-mediated increased invasiveness of cancer cells apparently requires BSP to have an active RGD sequence to bind to integrin $\alpha_v\beta_3$.

Integrins, particularly integrin $\alpha_v\beta_3$, modulate cancer cell invasiveness by directly interacting with MMP-2 (23), and BSP specifically binds to both active and pro-MMP-2 with nanomolar affinity (29). Because two other integrin $\alpha_v\beta_3$ -binding SIBLINGs, osteopontin and DMP1, did not stimulate the invasion of cancer cells, we hypothesized that BSP increases invasiveness by interacting with both MMP-2 and integrin $\alpha_v\beta_3$. BSP-enhanced SW-579 cell invasion was completely inhibited by the addition of 10 μ M 1,10-phenanthroline, a general MMP inhibitor (*P*<.001; Fig. 3). The role of MMP-2 in the BSP-enhanced cancer cell invasion was further investigated by use of a specific MMP-2 inhibitor (MMP-2 inhibitor I) and a monoclonal antibody against MMP-2. Both the specific MMP-2 inhibitor and the blocking antibody reduced the BSP-enhanced invasiveness of SW-579 cells to that of untreated control cells (*P*<.001; Fig. 3).

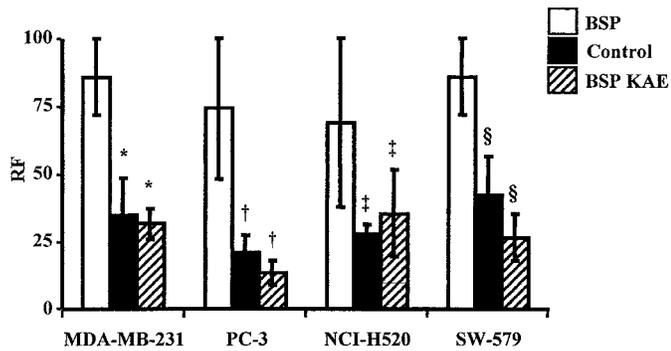


Fig. 2. Bone sialoprotein (BSP)-enhanced invasion and the RGD domain of BSP. MDA-MB-231, PC-3, NCI-H520, and SW-579 cells treated with 100 nM BSP or with 100 nM BSP-KAE (an inactive version of BSP in which the RGD domain was replaced with the chemically similar but inactive KAE tripeptide) were placed in the Matrigel-coated upper chamber of a Boyden chamber with a UV-opaque transwell insert. The lower chambers contained serum-free conditioned medium as a chemoattractant. The cells were incubated at 37 °C for 6–24 hours. Invasive cells that penetrated the Matrigel artificial basement membrane into the lower chamber were detected by calcein acetoxyethyl ester fluorescent dye, which is activated by living cells. The relative fluorescence (RF) in the lower chamber corresponds directly to the number of cells that have migrated through the Matrigel. The controls were untreated cells under the same culture conditions. The invasive activity of cells treated with BSP-KAE was similar to that of untreated cells. Relative fluorescence, which corresponds directly to the number of cells that migrated through the Matrigel, is as described in Fig. 1. Data are the means of quadruplicate samples, and **error bars** are 95% confidence intervals. A Mann–Whitney *U* test was used for the comparison of BSP-treated cells with either untreated control cells or BSP-KAE-treated cells. A *P* value of <.01 was obtained for MDA-MB-231 cells (*), PC-3 cells (†), NCI-H520 cells (‡), and SW-579 cells (§). All statistical tests were two-sided.

In Vitro Interaction of BSP, MMP-2, and Integrin $\alpha_v\beta_3$

Because the BSP-enhanced invasion can be blocked by interfering with the activity of either integrin $\alpha_v\beta_3$ or MMP-2 and because BSP can form a complex with integrin $\alpha_v\beta_3$ and a complex with MMP-2, we hypothesized that these three molecules form an RGD-dependent complex in which BSP acts as a bridge to link MMP-2 and integrin $\alpha_v\beta_3$. We tested this hypothesis with immunoprecipitation experiments using purified components. Integrin $\alpha_v\beta_3$ was incubated with immunoaffinity gel beads with covalently attached anti-integrin $\alpha_v\beta_3$ monoclonal antibodies to allow integrin binding. The beads were washed to remove unattached integrins, and the washed beads were then incubated with buffer alone or buffer containing recombinant BSP or recombinant BSP-KAE. After washing to remove unbound proteins, beads were incubated with soluble active MMP-2 or inactive pro-MMP-2. The beads were washed again, and then the amount of bound MMP-2 activity was measured by use of gelatin zymogram electrophoresis. No MMP activity was detected when integrin $\alpha_v\beta_3$ -free beads were used, which showed that the immunoprecipitation assay had a very low background (data not shown). Beads with bound integrin $\alpha_v\beta_3$ but no BSP bound a small but reproducible amount of pro-MMP-2 and an even smaller amount of active MMP-2 (Fig. 4, lane 2). This result confirms that of Brooks et al. (23) and suggests that some MMP-2 can bind directly to integrin $\alpha_v\beta_3$. Addition of BSP-KAE, which lacks an active integrin-binding RGD sequence, did not increase the binding between integrin $\alpha_v\beta_3$ and either MMP-2 or pro-MMP-2 (Fig. 4, lane 4). However, addition of BSP and then of MMP-2 (active MMP-2 or

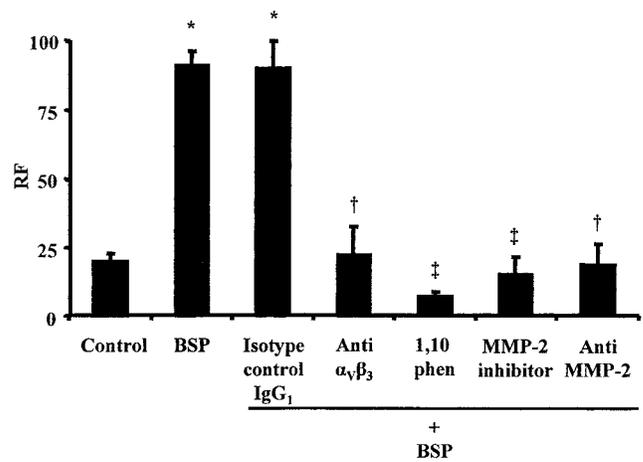


Fig. 3. Bone sialoprotein (BSP) activity and agents that block integrin $\alpha_v\beta_3$ or matrix metalloproteinase 2 (MMP-2) activities. SW-579 cells were placed in the Matrigel-coated upper chamber of a Boyden chamber with a UV-opaque transwell insert. The lower chambers contained serum-free conditioned medium as a chemoattractant. Invasive cells that penetrated the Matrigel artificial basement membrane into the lower chamber were detected by calcein acetoxyethyl ester fluorescent dye, which is activated by living cells. Cells were treated with isotype control immunoglobulin G₁ (IgG₁) (5 μ g/mL), anti-integrin $\alpha_v\beta_3$ monoclonal antibody (5 μ g/mL), anti-MMP-2 monoclonal antibody (5 μ g/mL), the general MMP inhibitor 1,10-phenanthroline (1,10 phen) at 10 μ M, or the specific MMP-2 inhibitor termed MMP-2 inhibitor I at 5 μ M for 20 minutes, before 100 nM BSP was added to the inserts. The cells were incubated at 37 °C for 24 hours. Interfering with the functions of MMP or integrin negated BSP-enhanced Matrigel invasion. Relative fluorescence (RF) is as described in Fig. 1. Data are the means of quadruplicate samples from a representative experiment, and **error bars** are 95% confidence intervals. A *P* of less than .001 was obtained for multiple comparisons by use of one-way analysis of variance. Each group was also individually compared with untreated control group by use of the Dunnett test. *, *P*<.001 compared with untreated control cells; †, *P*<.001 compared with cells treated with isotype control IgG₁ and BSP; ‡, *P*<.001 compared with BSP-treated cells. All statistical tests were two-sided.

pro-MMP-2) to integrin $\alpha_v\beta_3$ -coated beads increased MMP activity associated with the beads, indicating that BSP stimulated the formation of a complex between integrin $\alpha_v\beta_3$ and MMP-2, presumably by linking the two proteins (Fig. 4, lane 3).

To investigate whether the complex of BSP, integrin $\alpha_v\beta_3$, and MMP-2 also occurs on living cells, we used flow cytometry, SW-579 cells, and purified active MMP-2 and pro-MMP-2 that had been covalently labeled with AlexaFluor-488. SW-579 cells were treated with anti-integrin $\alpha_v\beta_3$ monoclonal antibody or with an isotype control IgG₁ and then washed. These cells were incubated with buffer alone, buffer containing BSP, or buffer containing BSP-KAE, washed again, and then incubated with labeled pro-MMP-2. Flow cytometry was used to determine the amount of labeled pro-MMP-2 bound to these cells relative to that bound to untreated cells. Addition of BSP produced a 43% increase in the signal of labeled pro-MMP-2 bound to SW-579 cells compared with that of untreated cells (Fig. 5, A), whereas addition of BSP-KAE produced no change in this signal (Fig. 5, B). Addition of anti-integrin $\alpha_v\beta_3$ monoclonal antibodies almost completely blocked the binding of the labeled pro-MMPs to SW-579 cells compared with that of untreated control cells (Fig. 5, C) or cells treated with isotype control IgG₁ (Fig. 5, D). When labeled active MMP-2 was used for the binding analysis, BSP produced a 23% and 22% increase in signal of labeled MMP-2 bound to SW-579 cells compared with untreated control or BSP-KAE-treated cells, respectively (data not shown). Essen-

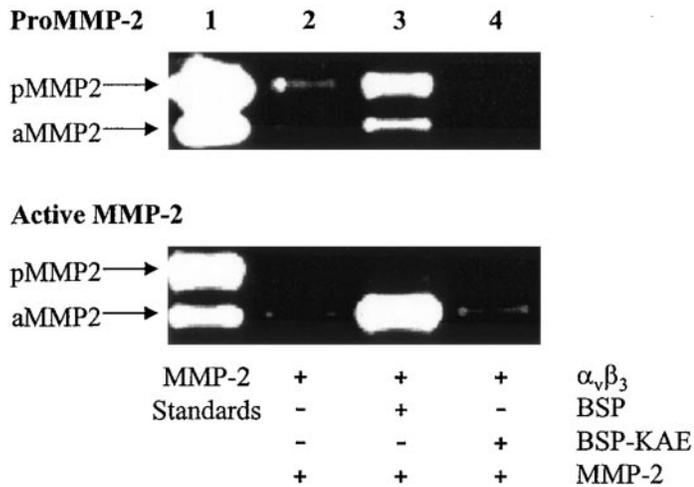


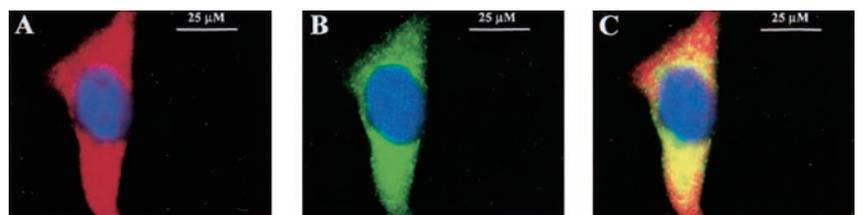
Fig. 4. Immunoprecipitation experiments and complexes of integrin $\alpha_v\beta_3$, bone sialoprotein (BSP), and matrix metalloproteinase 2 (MMP-2). Integrin $\alpha_v\beta_3$ was first bound to an anti-integrin monoclonal antibody covalently attached to immunoaffinity gel beads. After washing, the beads were incubated with buffer alone or buffer containing 500 nM BSP or 500 nM BSP-KAE, washed, and subsequently treated with recombinant pro-MMP-2 or active MMP-2. The washed samples were then separated by electrophoresis on 10% gelatin gels and examined by gelatin zymography. Note that the addition of BSP (lane 3) but not BSP-KAE (lane 4) enabled both pro-MMP-2 (upper panel) and active MMP-2 (lower panel) to be immunoprecipitated with the integrin-bound beads. Trace amounts of both pro-MMP-2 and active MMP-2 were observed without addition of BSP (lane 2), as described by Brooks et al. (23). **Arrows** = pro-MMP-2 (pMMP2) and active MMP-2 (aMMP2) and identify the migration positions of MMP standards in lane 1.

tially the same results for both active MMP-2 and pro-MMP-2 were obtained with PC-3 cells (data not shown).

Fluorescent immunocytochemistry experiments showed that BSP, MMP-2, and $\alpha_v\beta_3$ integrin co-localized in single cells. SW-579 cells were first treated with BSP overnight and then treated with MMP-2 for 20 minutes. BSP and MMP-2 were detected at the same locations in these cells (Fig. 6, A–C). Neither BSP nor MMP-2 was detected on untreated cells or cells treated with BSP-KAE (data not shown), suggesting that BSP rapidly localizes MMP-2 to the cell surface *in vitro* by binding to integrin $\alpha_v\beta_3$.

Finally, to verify that the complex of BSP, MMP-2, and integrin $\alpha_v\beta_3$ may occur naturally *in vivo*, *in situ* hybridization experiments were performed on serial paraffin sections of human papillary thyroid carcinomas. The purple-blue cytoplasmic staining with antisense probes verified that all four mRNAs are expressed in the same cells and/or areas of tissue (Fig. 7), showing that all the components of the complex are synthesized simultaneously by cells. Hybridization with similar amounts of the sense probes revealed no signal (data not shown).

Fig. 6. Localization of bone sialoprotein (BSP) and matrix metalloproteinase 2 (MMP-2) on SW-579 thyroid cancer cells. Cells were treated first with BSP and then with pro-MMP-2. The cells were incubated with a polyclonal antibody against BSP and a monoclonal antibody against MMP-2, and both proteins were detected by indirect immunofluorescence with Rhodamine Red-conjugated AffiniPure goat anti-rabbit immunoglobulin G (IgG) for BSP and Cy2-conjugated AffiniPure goat anti-mouse IgG for MMP-2. The red color in panel



A shows the location of BSP, the green color in **panel B** shows the location of MMP-2, and the yellow color in **panel C** shows that the two proteins co-localize. 4',6-Diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain (blue color). **Scale bar** = 25 μm .

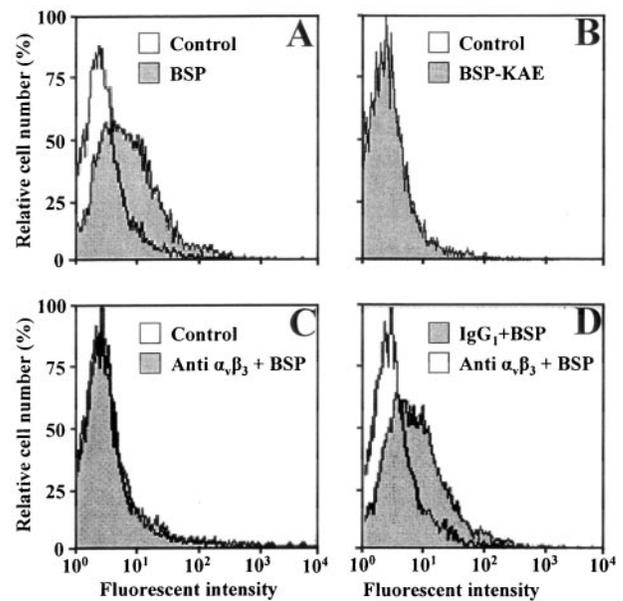


Fig. 5. Bone sialoprotein (BSP)-enhanced binding of fluorescently labeled pro-matrix metalloproteinase 2 (pro-MMP-2) to SW-579 cells. Pro-MMP-2 was labeled with Alexa Fluor-488 and incubated with cells treated as indicated or untreated cells (Control). **A**) Pretreating the cells with BSP (shaded area) increased the amount of labeled pro-MMP-2 bound to the living cells compared with untreated cells. **B**) Pretreating cells with BSP-KAE (shaded area) showed no increased binding of labeled pro-MMP-2 compared with that of untreated cells (open area). **C**) Blocking cell-surface integrin $\alpha_v\beta_3$ with an anti-integrin monoclonal antibody (shaded area) also completely blocked the BSP-enhanced binding of the labeled pro-MMP-2 (open area). **D**) Treating cells with a nonimmune isotype specific immunoglobulin G₁ (IgG₁) had no effect on the ability of BSP to enhance the binding of pro-MMP-2.

DISCUSSION

Cellular invasion requires dynamic coordination of many cellular components, including cell adhesion molecules and proteolytic agents. MMPs and integrins participate in degradation of the basement membrane and, consequently, in the invasion of cancer cells. Several studies have reported that increased activation of MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, and/or MMP-15 is associated with tumor cell invasion (23,38–44). Two studies have demonstrated that the progression of various cancers is also associated with the overexpression of integrins $\alpha_v\beta_3$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, and/or $\alpha_2\beta_3$ (23,45). Finally, several colocalization studies have reported that integrin $\alpha_v\beta_3$ may function not only as an adhesion and/or migration receptor but may also activate and properly distribute proteases that degrade the extracellular matrix during invasion (23,46,47).

Because it is overexpressed in malignant tissues, BSP may play a role in the progression and invasion of a number of

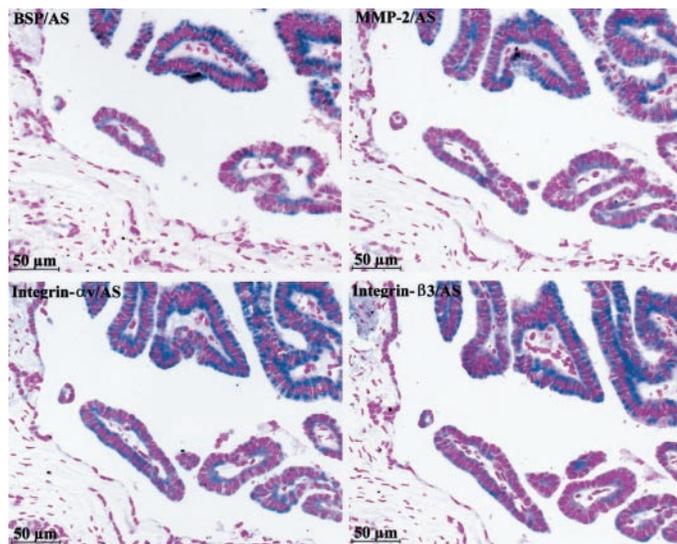


Fig. 7. mRNA for bone sialoprotein (BSP), matrix metalloproteinase 2 (MMP-2), and both chains of integrin $\alpha_v\beta_3$ in sections of an invasive thyroid papillary carcinoma. Deparaffinized sections of an invasive thyroid papillary carcinoma were incubated with digoxigenin-labeled antisense probes specific for BSP, MMP-2, the α_v chain, and the β_3 chain. Indirect localization of the probes was determined with anti-digoxigenin antibodies and colorimetric staining. Cytoplasmic purple-blue color indicates that mRNAs for BSP (upper left), MMP-2 (upper right), α_v chain (lower left), and β_3 chain (lower right) are in the same cells, suggesting that all proteins required for formation of complexes of integrin $\alpha_v\beta_3$, BSP, and MMP-2 are likely to be expressed in the same cells. Fast red was used as a nuclear counterstain (nuclear red color). Scale bar = 50 μm .

osteotropic cancers, including breast, prostate, lung, and thyroid cancers (14–17,20). Addition of BSP stimulates the *in vitro* migration of breast cancer cells via a mechanism involving integrin $\alpha_v\beta_3$ (48,49). Strong and specific *in vitro* interactions (with nanomolar affinity) have been described (29) between three members of the SIBLING family (BSP, osteopontin, and DMP1) and specific MMPs (MMP-2, MMP-3, and MMP-9, respectively). Thus, the combination of BSP, MMP-2, and integrin $\alpha_v\beta_3$ appears to play an important role in cancer cell invasion. This study demonstrated that BSP, but not osteopontin or DMP1, increased the Matrigel invasiveness of a large subset of breast, prostate, lung, and thyroid cancer cell lines. Addition of BSP-KAE, a recombinant form of BSP in which the RGD sequence was replaced with KAE, or addition of an antibody that blocks BSP binding to integrin $\alpha_v\beta_3$ through RGD sequence blocked all BSP-enhanced invasive activity, suggesting that BSP acts through this integrin. The BSP-enhanced invasion by these cells was also inhibited by specific chemical inhibitors of MMP-2 and by an antibody for MMP-2. Formation of a complex containing BSP, integrin $\alpha_v\beta_3$, and MMP-2 was demonstrated by immunoprecipitation experiments, immunofluorescence experiments, and flow cytometry. These results suggest that cells use BSP as a bridge to link MMP-2 to its cell surface receptor, integrin $\alpha_v\beta_3$, which thereby increases their ability to invade basement membranes and other connective tissues. Fig. 8 shows BSP with an intact RGD bridging MMP-2 to its cell-surface receptor, integrin $\alpha_v\beta_3$. When the integrin-binding RGD sequence is replaced with the chemically similar but inactive KAE sequence, MMP-2 may still bind to the BSP-KAE protein, but the complex between MMP-2 and BSP-KAE does not interact with cell-surface integrin.

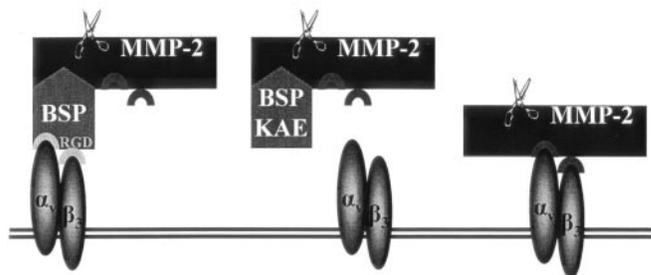


Fig. 8. Proposed trimolecular complex of bone sialoprotein (BSP), matrix metalloproteinase 2 (MMP-2), and integrin $\alpha_v\beta_3$. (Left) BSP with an intact RGD domain acts as a bridge between MMP-2 and integrin $\alpha_v\beta_3$. (Middle) BSP-KAE, mutant BSP in which the RGD is replaced with KAE, can bind to MMP-2, but the complex does not interact with the integrin $\alpha_v\beta_3$ heterodimer. (Right) As shown in Fig. 4 and by others (23), some MMP-2 may also interact with integrin $\alpha_v\beta_3$ even in the absence of BSP. Double line = cell membrane; scissors = active site of MMP-2.

Because BSP enhances the attachment and migration of several cancer cell lines, we also tested the effect of two other members of the SIBLING family, osteopontin and DMP1, that support cell attachment. Osteopontin also supports migration (50). For all 16 cell lines tested, neither osteopontin nor DMP1 increased the invasiveness of these cells through Matrigel, even though the increased expression of these proteins in various cancers has been reported (20,51–53). We have recently shown *in vitro* that osteopontin and DMP1 bind specifically to MMP-3 and MMP-9, respectively, with nanomolar affinity but do not bind to MMP-2 (29). Because the Boyden chamber cell invasion assay can detect differences only in those functions that result in a net change in rate-limiting steps in the *in vitro* invasion process (54), it is reasonable to conclude that the binding and possible activation of MMP-2 by BSP overcomes such a rate-limiting step for the cell lines tested, whereas cell attachment alone or the binding and activation of MMP-3 by osteopontin or MMP-9 by DMP1 does not overcome such a step.

In conclusion, we have shown that recombinant BSP can enhance the invasiveness of some, but not all, breast, prostate, lung, and thyroid cancer cell lines in a modified Boyden chamber assay through formation of an RGD-dependent complex with MMP-2 and integrin $\alpha_v\beta_3$.

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